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J. Histochem. Cytochem 11: 24-34 (1963)

QUANTITATIVE APPROACHES IN THE HISTOCHEMISTRY OF MUCOPOLYSACCHARIDES*

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Received for publication July 9, 1962

Although the subject matter of histochemistry can be defined as the "identification and localization of chemical substances in the tissues on a [illegible] level" (20), many histochemists will prefer to give their discipline a wider scope and include as another important aim, that of obtaining quantitative information. Quantitation seems to be of paramount importance in histochemistry for any study of metabolic activity or biological significance of a given constituent of a cell or tissue. Several examples in which this goal of quantitative estimation on the tissue level has been practically realised, such as microspectrophotometric measurements of nucleic acids or micro- and ultramicrochemical determination of enzyme activities or of other substances, illustrate clearly the significant contributions of quantitative histochemistry.

Mucopolysaccharides[1] do not seem yet to belong to those substances for which this important goal has been reached or even successfully approached. The contrast between the great number of staining methods and their modifications and the scarcity of pertinent information on the occurrence and quantitative distribution of various mucopolysaccharides in different tissues characterizes adequately the present state of affairs. Consequently a review of the quantitative aspects of these methods cannot offer more than to point out shortcomings and limitations and sketch some attempts which eventually might provide us with better tools in the future.

In this presentation the quantitative aspects of some staining type of methods for the histochemical demonstration of mucopolysaccharides will be reviewed, based mainly on our work with Dr. E. A. Balazs using cationic dyes. Some considerations relative to the problems of fixation will necessarily precede this discussion. In the second part, some attempts of applying microchemical methods to the analysis of mucopolysaccharides on the tissue level will be presented, worked out in collaboration with Dr. S. Gardell.

* From the Symposium presented at the 13th Annual Meeting of the Histochemical Society, supported in part by grant G-21841 from the National Science Foundation.

[1] Since the descriptive term "mucopolysaccharide" has been used throughout this symposium, it will be retained in this paper; one should note that recently the use of the name "glycosaminoglycan" has been suggested for polysaccharides containing amino sugars (23).

FIXATION OF MUCOPOLYSACCHARIDES

The classical requirements of fixation in histological terms include instant immobilization of the metabolic events and their substrates, preservation of the spatial relationship of the cell and tissue components, prevention of physical, chemical and autolytic artefacts, stabilization against subsequent steps of treatment and promoting (or non-interference with) the staining of the various tissue structures. To fulfill these criteria in the case of mucopolysaccharides appears more difficult than generally appreciated. One has only to think of the properties of a substance like hyaluronic acid, its easy solubility and occurrence in the tissue in a highly hydrated state, to understand that most of the commonly used routine fixatives will not adequately serve the purpose. An example of a generally applied fixative which is for mucopolysaccharides entirely unsatisfactory is a solution of formaldehyde in water, extracting these substances from the tissue to considerable extent. "Fixation" of a tissue like umbilical cord, vitreous body, rooster comb or many pathological tissues in a formaldehyde solution will result in the accumulation on the bottom of the vessel of a highly viscous gel, indicating that part of the mucopolysaccharide has gone, and with it the hope to demonstrate these substances properly and quantitatively. Although this phenomenon might be indicative of the presence of mucopolysaccharide in the tissue—recently even described as an useful screening test (38)—this procedure will certainly not qualify as histochemical. The partial preservation of mucopolysaccharides upon formaldehyde treatment seems not to be based on any real fixation of these substances but rather on the reaction of formaldehyde with structurally

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associated proteins. Since this latter interaction results in a lowering of the isoelectric point, this fixation will interfere with subsequent staining of mucopolysaccharides with cationic dyes through an overall increase of basophilia. The above facts indicate that an aqueous solution of formaldehyde is undoubtedly an undesirable fixative for mucopolysaccharides, in spite of some contrary opinions (33).

The use of alcohol or other dehydrating agents—alone, with addition of salts or as part of fixative mixtures—will tend to prevent solubilization of mucopolysaccharide. This will mainly be based on their precipitation and leave them chemically unaltered. Such a precipitation is usually not irreversible and consequently resolubilization can occur during subsequent steps of the histological processing in water containing media. The marked shrinkage of alcohol-fixed tissues after routine processing is likely to introduce changes in the structural relationship and might result in misleading localization.

The use of acids and acid containing fixatives for "mucins" might be traced back to the common use of acetic acid as a simple test for biological substances a century ago (30), having its analog in the laboratory test of "mucin clot" formation of synovial fluid. Under these conditions mucopolysaccharides are precipitated as a complex with the protein components present. In this coprecipitation the nature of the mucopolysaccharides is somewhat altered, since their anionic groups are involved in the complex formation with protein. Depending on the strength of the linkage and on subsequent treatment, such "mucin clot formation within the tissue" might to some extent interfere with the use of cationic dyes for the demonstration of mucopolysaccharides. The general recommendation of Carnoy's fluid, in which the effects of both acetic acid and alcohol are combined, would indicate that little loss of mucopolysaccharide occurs with this and similar procedures, although this is not supported by any quantitative analysis.

Another principle resulting in decreased solubility of mucopolysaccharides is the use of metal cations or other cationic substances in aqueous or alcoholic solution. Examples are basic lead acetate (22), barium hydroxide (12), lead nitrate and calcium acetate (31) or quaternary ammonium compounds (40). Good results have been obtained [illegible] such alcoholic fixatives in freeze-substi- [illegible] procedures seem to have a sound chemical basis in view of the somewhat analogous procedures used for precipitation of mucopolysaccharides in preparative work, such as alcohol in combination with barium (24, 17) or calcium (34) and long-chain aliphatic quaternary ammonium compounds (40, 41). Obviously these cationic substances act in precipitating the mucopolysaccharides as their salts. In histochemical terms this means that the reactive anionic groups of mucopolysaccharides are involved and in principle not available for interaction with other cationic substances like cationic dyes. The common experience of decreased metachromasia after prolonged basic lead acetate fixation, restored after acid treatment, bears out this point clearly. As in the case of alcohol, the use of these substances results in a marked shrinkage of the tissue and often distortion of its finer structure.

The above considerations indicate that "fixation" of mucopolysaccharides will mainly consist of their precipitation within the tissue structures. Undesirable effects as physical distortion due to shrinkage or a considerable degree of reversibility with danger of subsequent losses are minor in comparison with the disadvantage of the fixative reacting with the same chemical group which subsequently has to be used for the histochemical demonstration of the mucopolysaccharides. Depending on the concentration of the reagents used the cationic compound employed in precipitation might subsequently be replaced by the dye cation, reasons why in practice the use in fixation of the above mentioned salts yields qualitatively satisfactory results.

From these and further considerations it will appear that the interaction of cationic dyes with mucopolysaccharides is essentially comparable to their interaction with cations like barium, calcium or quaternary ammonium compounds. Appreciating this analogy would indeed mean that a fixative effect can be achieved with the immediate use of the cationic dye itself, obviating the replacement of one cation for another. This in fact can be realized with very satisfactory results with the use of fresh frozen sections, stained immediately with cationic dye solutions of sufficiently high concentration to precipitate the mucopolysaccharides *in situ* (43, 7). This procedure, avoiding shrinkage and exposure of the mucopolysaccharides to other solvents than the dye, gave in our hands results superior to those obtained on fixed and paraffin embedded material. Although exposing the sections to osmium tetroxide vapors improves cytological details, such treatment detracts from the main advantage of this procedure, i.e. avoiding chemical alterations, which is an absolute prerequisite for the evaluation of the quantitative nature of the interaction of mucopolysaccharides with cationic dyes.

CATIONIC DYES FOR THE DEMONSTRATION OF MUCOPOLYSACCHARIDES AND THE STOICHIOMETRY OF THE REACTION

It would appear that practically all of the staining methods for mucopolysaccharides depend on the use of cationic dyes or cationic complexes colored subsequently by other procedures. In this review only the simple cationic dyes will be discussed, since only in this case data are available on the quantitative nature of their interaction with mucopolysaccharides, supplemented by chemical studies. These dyes usually exhibit metachromasia, which will not be considered here, having been reviewed recently on several occasions (39, 26, 9). In fact the phenomenon of metachromasia, i.e. the change of the absorption spectra, is not of fundamental importance from a quantitative point of view and its study in solutions seems to have detracted attention from the phenomena of precipitation. These have been known to the old histologists and classified clearly in respect to histological staining as "Niederschlagsfärbung" (35). More recently, studies of this aspect of the reaction of cationic dyes have been initiated by Balazs and collaborators (4, 5, 6, 44). Although the finally adopted method of assay of the dyebinding in tissues is more a microchemical procedure, it has direct relevance on the conditions under which cationic dyes should be used for optimal histochemical staining of mucopolysaccharides. In many ways these studies represent tests of histochemical applicability and they will be therefore briefly summarized below.

If a solution of an anionic polysaccharide is mixed *in vitro* with a cationic dye such as azure A in sufficient amount and concentration, a metachromatic precipitate will form which can be separated by centrifugation and the amount of dye bound estimated by measuring the extinction of the supernatant free dye. The conditions under which such a precipitate is obtained instead of the formation of a soluble-metachromatic complex, have been given in detail elsewhere (5, 44). When this quantitative assay is applied to purified mucopolysaccharides and the dyebinding compared with their amount and chemical composition, one finds a quantitative relationship between the number of dye molecules bound and the number of anionic groups per disaccharide unit (Fig. 1). This means not only that mucopolysaccharides can be precipitated by cationic dyes, but also that they are precipitated in a stoichiometric way, each dye molecule occupying one negative site.

Similar studies have also been carried out on extracts of connective tissues, containing mucopolysaccharides, and on such tissues directly. In

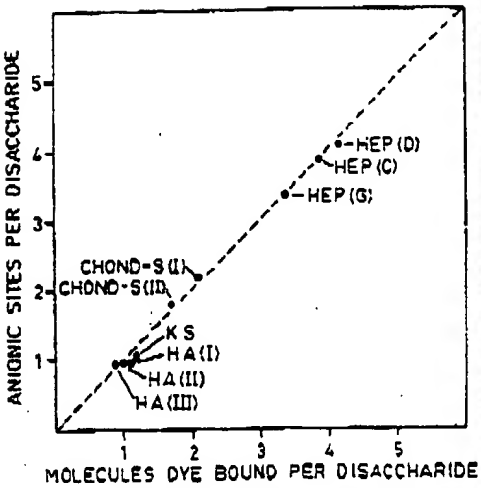
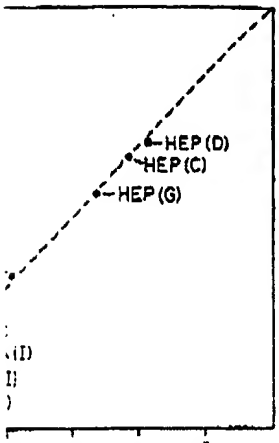


FIG. 1. Correlation between the amount of cationic dye bound and the number of anionic sites per disaccharide unit of various mucopolysaccharides. Various batches of Na or K salts of mucopolysaccharides (HA I, II, III, hyaluronic acid; KS, keratan sulfate; CHONDR-S I, II, chondroitin sulfate, and HEP, C, D, G, heparin) were used in aqueous solutions (pH 6 to 7). Azure A (1 to 0.1 *mM*) was used as cationic dye. The dotted line represents the theoretical one-to-one ratio between anionic sites and the dye molecules bound. (Modified from Szirmai and Balazs (44), with permission of the Gustav Fischer Verlag, Jena.)

the latter case unfixed frozen sections are placed into the dye bath and are removed from it by centrifugation after equilibrium has been reached. In this way we attempted to investigate how far the stoichiometry also holds for the native mucopolysaccharide in the tissue. The results of such studies (5, 6, 46, 47) indicate that crude extracts of mucopolysaccharide from tissues like rooster comb or nasal septum cartilage bind the cationic dye proportionally to the mucopolysaccharide content within an error of up to 10%. Although no quantitative studies have been made on the contribution of substances other than mucopolysaccharides to the dyebinding of such extracts, it seems likely that the somewhat higher values will be due to binding by the carboxyl groups of the proteins present. The ratio of bound dye to the anionic groups of mucopolysaccharides in the case of whole tissue sections usually exceeds unity further than in extracts, up to 1.2 or 1.3 in the case of nasal septum cartilage (46, 47). This clearly indicates that anionic groups other than those of mucopolysaccharides also participate in the dye-binding, and in addition to proteins evidently nucleic acids also contribute to the total value



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(45, 28, 48). Purification of crude homogenates of nasal septum cartilage by peptic and tryptic digestion and dialysis showed that removal of protein —even if incomplete—will bring the bound dye/anionic group of mucopolysaccharide ratio close to unity (46, 47).

From the above studies and other considerations we are inclined to conclude that the interaction of cationic dyes like azure A—as well as any other monobasic cationic dye—with mucopolysaccharides results, under suitable conditions, in the formation of a dye-polyanion complex, in which the components are present in a stoichiometric ratio. The same conclusion in respect to quantitative relationship seems also to be valid when dyebinding is carried out on unfixed tissue sections. Whereas the contribution of anionic groups other than those of mucopolysaccharides in the tissue will correspondingly increase the final value of dyebinding, in some instances microscopic evaluation might distinguish between the various components involved. These data also suggest that in the cases studied the anionic groups of the native mucopolysaccharide are free or in any case not participating in any interaction which could not be displaced by the cationic dye under the conditions used.

The practical considerations emerging from these studies in respect to quantitative histochemistry are twofold. First, establishment of the stoichiometry of this interaction validates the use of the dyebinding assay as a micro- or ultramicrochemical method, applicable to unfixed fresh or frozen-dried tissue sections and providing an overall estimate of the free anionic groups in the tissue. Under certain conditions and depending on the mucopolysaccharide content of the tissue in respect to the other constituents, the values might in practice give an approximate quantitative measure of the total mucopolysaccharide of a tissue. Some applications of this method have been made recently and showed satisfactory agreement of the results with chemical data (46, 47, 36). Secondly, it would appear that the establishment of this stoichiometry makes this reaction applicable to direct measurement of the metachromatic complex in the tissue using the technique of microspectrophotometry. Further studies in this direction are necessary, such as concerning conformity to the Beer-Lambert law of the metachromatic complex, elaboration of a suitable microscopic model for quantitation in terms of concentration of the free anionic groups, finding a substitute of suitable refractive index for water as mounting medium, which seems to be necessary for the maintenance of the spectral characteristics of the complex, etc. Also fixation in the usual sense should be avoided in such work. In fact approaches along these lines have been made by several investigators in the past (11, 10, 27). Utilizing the advantages of the stoichiometric nature of the reaction should make such efforts even more valuable in future studies.

Although the above considerations might indicate some rewarding possibilities for the use of cationic dyes for the quantitative estimation of mucopolysaccharides on the tissue level, some shortcomings such as the limited specificity due to participation of other polyanions constitute definite drawbacks. Another serious limitation lies in the difficulty in distinguishing the various types of mucopolysaccharide using cationic dyes. Many attempts, mostly on somewhat empirical basis and with insufficient chemical control, have been made in this direction in the past. To quote only a few examples, differentiation has been attempted through variation of the alcohol concentration of the dye bath (42), the suppressing effect of different salt concentrations (8, 29) or the suppressing effect of low pH on the staining of various mucopolysaccharides (11). Although such procedures might be theoretically justified, they also raised many controversial points and might have severe limitations in practice (39). An illustration of such problems in relation to the effect of the pH on the dyebinding and metachromatic staining will be given below.

Since the interaction of cationic dyes with polyanions is of an electrostatic nature, it will obviously depend on the hydrogen ion concentration of the medium and the pK values of the anionic groups involved. This is clearly illustrated by the effect of the pH on the dyebinding of hyaluronic acid and chondroitin sulfate (Fig. 2). Hyaluronic acid, containing only one carboxyl group per disaccharide unit, will bind one dye molecule at neutral pH. Lowering the pH will gradually decrease the dissociation of the carboxyl group and suppress the dyebinding markedly below pH 3. In contrast, chondroitin sulfate binds two dye molecules per disaccharide unit at neutral pH, with both carboxyl and sulfate participating in the binding. The pK of these two charged groups being different, the lowering of the pH will first affect the carboxyl group, which will be unable to bind dye below pH 2, where the sulfate group is still nearly completely dissociated. The dyebinding at pH values of 1 to 2 is due practically entirely to the sulfate groups. This reduction of the dyebinding to about half at this low pH—or decrease of staining intensity in contrast to complete suppression in case of non-sulfated mucopolysaccharides would thus appear as an

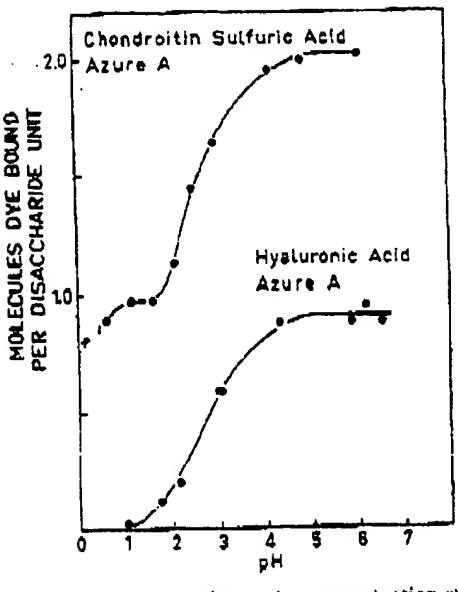

FIG. 2. Effect of hydrogen ion concentration on the dyebinding of chondroitin sulfate and hyaluronic acid. Azure A was used as in Fig. 1, the solutions were adjusted to various pH's with HCl. (From Szirmai and Balazs (44), with permission of the Gustav Fischer Verlag, Jena.)

useful mode of distinction between sulfated and non-sulfated mucopolysaccharides.

Many connective tissue structures show such an abolished staining with cationic dyes at low pH values, often interpreted—in full agreement with the above considerations—as an indication of the presence of a non-sulfated mucopolysaccharide. Such marked loss of the dyebinding and metachromatic staining has been observed in previous studies of the nasal septum cartilage of the steer or horse (44, 46, 47, 15). In this tissue the subperichondrial layer ("first layer") and in old animals the central part of the cartilage ("third layer") show a nearly complete suppression of the staining with azure A at pH 2, compared with the staining at pH 7 (Fig. 3). The interpretation of these differences among the various layers of this cartilage proved to be more complicated than should have been expected on the basis of the above considerations. For a further study of this phenomenon direct chemical analysis of pooled samples of dissected layers has been carried out as well as the dyebinding determined quantitatively throughout the various layers (46, 47). Dyebinding was measured on serial cryostat sections [illegible] a cylinder perpendicularly to the surface of the septum, alternate groups being used for histochemical control and dyebinding assay at pH 2 and 7 respectively. The results of such application of the dyebinding are illustrated in Fig. 4. They show a gradual increase of the concentration of free anionic sites towards the central part of the cartilage, with a slight decrease in the third layer. Assuming the presence of a sulfated mucopolysaccharide like chondroitin sulfate, one would expect a decrease of the dyebinding at pH 2 to about half of the value found at pH 7. Whereas in the second layer the ratio of the dyebinding at pH 2/pH 7 is indeed close to the expected 0.5, it is less than 0.1 in the first layer and only about 0.25 for the third layer. Chemical analysis of the isolated layers showed however that in all three layers the total hexosamine, hexuronic acid and sulfate content were reasonably close to equimolar and compatible with assuming most of the mucopolysaccharide to be chondroitine sulfate, although the values were quite low in the first layer. These data suggested by no means evidence for the presence of a non-sulfated mucopolysaccharide, which could have been inferred from the above effect of the pH. Analysis of the isolated layers of the nasal septum cartilage further showed a very high protein content in the first and a quite high protein content in the third layer, in comparison with the second layer. This suggested that the suppression of the dyebinding at low pH might be due to the relatively large amount of proteins in these areas. That the proteins in cartilage tissue in fact do interfere with the binding of the dye has been further substantiated by studies using deproteinization and formaldehyde treatment of cartilage homogenates, both resulting in the increase of the dyebinding at pH 2, whereas practically no effect was observed on the dyebinding at pH 7 (46, 47). These experiments would indicate as one of the possible explanations a competitive effect of the cationic groups of proteins, relatively increasing by lowering the pH. Several observations in the past suggested such an interfering effect on the metachromatic staining in solutions (21, 13, 25). In tissue similar effects might occur, for which further evidence was obtained in regard to microscopic structures of the cartilage ground substance in histochemical and interferometric studies (14, 15, 16).

Utilization of the effect of pH in differential histochemical staining with cationic dyes appears to be valid only under conditions where the above described interfering effect of proteins can be excluded, which will hardly be the case in any tissue without detailed chemical analysis. Other [illegible] of quantitatively distinguishing sulfated and non-sulfated mucopolysaccharides, such as differential removal of the dye by salt solutions, might also require further careful com-

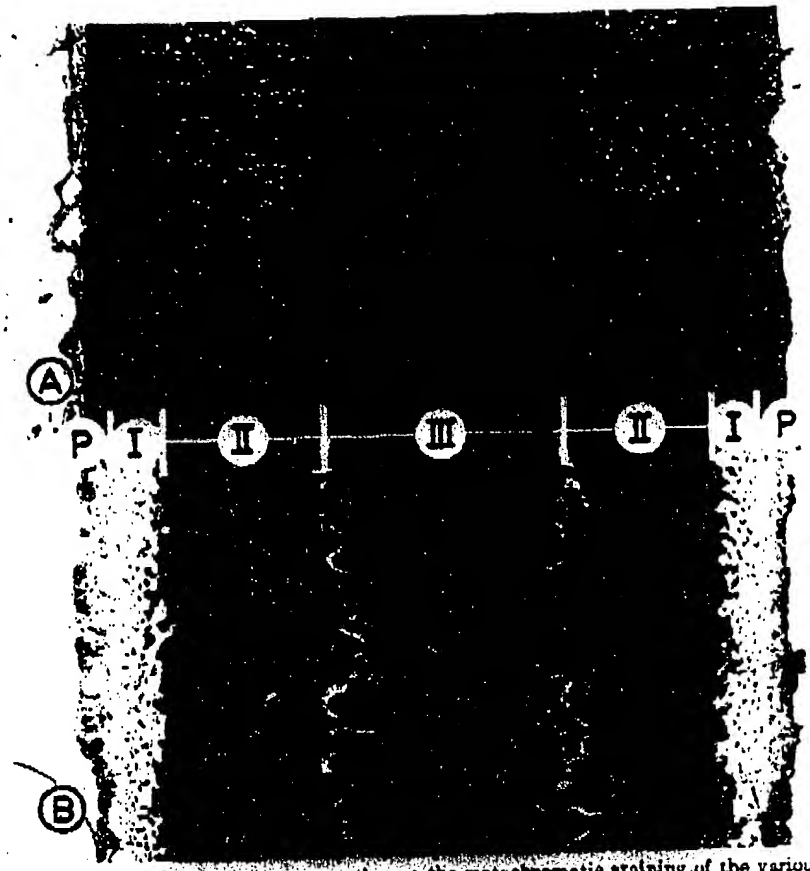

FIG. 3. Effect of hydrogen ion concentration on the metachromatic staining of the various layers of the nasal septum cartilage (horse, > 8 years old). Unfixed frozen sections stained with an aqueous solution of azure A (2 *mM*) and mounted in water. *A*. Stained at pH 0.5; *B*. Stained at pH 2.0, adjusted with HCl. P, perichondrium; I, first layer; II, second layer; III, third layer. (From Szirmai and Doyle (47).)

parison with chemical data. Although from biochemical work useful indications regarding the critical salt solubility of cation-polyanion complexes might be derived (41), and *in vitro* studies also have provided ample data on salt effects on metachromasia (5), such data apply mainly to purified substances. In tissues proteins are always present and might modify the dissociability of the the cation mucopolysaccharide complexes.

In spite of the established stoichiometric character of the interaction of cationic dyes with mucopolysaccharides—provided the necessary criteria regarding fixation and other conditions are fulfilled—their use for the quantitation of the various mucopolysaccharides seems as yet to be limited. In some tissues and under certain conditions cationic dyes will give a reasonably accurate estimate of the total free anionic sites of muco-

of distinguishing quantitatively the various substances within this group. In cases where one is dealing with one type of mucopolysaccharide only, or where differentiation between various mucopolysaccharides is not required, cationic dyes certainly have potentialities worth further exploration. As a histochemical staining method they seem to be superior to some other procedures such as using colloidal iron, alcian blue or one of their many modifications, which—although probably basically similar—have not been investigated in respect to their quantitative nature and are often carried out at pH values where no optimal reaction can be expected.

MICROCHEMICAL DETERMINATION OF MUCOPOLYSACCHARIDES IN TISSUES

The example of the nasal septum cartilage, quoted in the previous section, where several

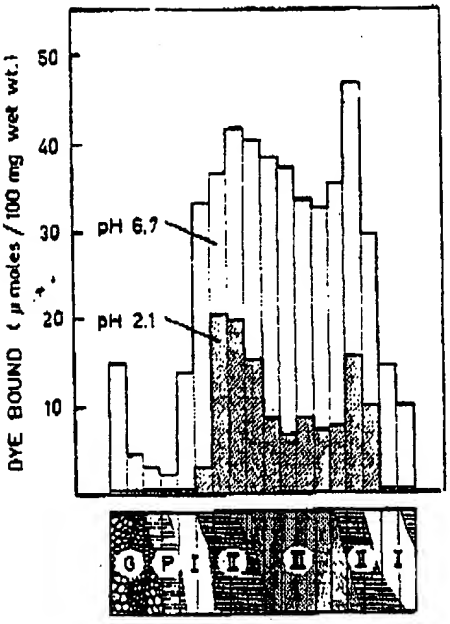

FIG. 4. Quantitative assay of the dyebinding on serial unfixed frozen sections of the nasal septum cartilage (horse, > 8 years old). Azure A (0.2 *mM*) in aqueous solution at pH 6.7 and 2.1 respectively, the latter adjusted with HCl. Histological reconstruction (bottom) corresponds to the layers shown in Fig. 3. (From Szirmai and Doyle (47).)

layers could be distinguished on the basis of histochemical staining or quantitative assay of the binding of cationic dyes, illustrates clearly that such methods might reveal more differences than they can explain in terms of chemical composition. Further analysis of isolated layers of the nasal septum cartilage showed differences among the three layers in the glucosamine/galactosamine ratio (46, 47), and it became apparent that only quantitative fractionation of the various mucopolysaccharides would provide further information. A method was needed sensitive enough to deal with a few micrograms of mucopolysaccharide and recovering quantitatively the various fractions from the tissue structures suitably isolated. The method of fractionation using quaternary ammonium compounds like cetylpyridinium chloride, based on the studies of Scott (40, 41), seemed to have potentialities for such an approach. This principle of fractionation has been adapted by many workers in the biochemical field, but so far the lowest limit at its best was in the milligram order of magnitude (37). Preliminary experiments by Gardell and Scott (18, 1) showed the possibility of recovering pure mucopolysaccharide from a mixture down to 25–50 μg. In a joint effort with Dr. Gardell and his group we attempted to develop a method according to this principle on the microgram scale. The outline of the procedure and some of its results are presented here merely to illustrate the possibilities and present limitations of this approach; the method has been published in a preliminary form (2) and will be dealt with in detail elsewhere (3).

The method is based on the principle of precipitating the mucopolysaccharide mixture from the solubilized tissue by cetylpyridinium chloride and stepwise fractional elution with solutions of neutral salts of increasing concentration using a cellulose column as support. Briefly, the subsequent steps are as follows. The tissue sample consists of one or several tissue sections obtained by serial cryostat sectioning or of microdissected portions of frozen-dried sections, obtained according to the procedures developed by Lowry (32). The tissue samples are, depending on their mucopolysaccharide content, of the order of magnitude from 50 to several 100 μg. weighed with sufficient accuracy by the use of an electrobalance (Cahn Instrument Co., Paramount, California). Placing the weighing chamber of the balance into the cryostat allows also immediate measurement of the wet weight and calculation of the water content of the samples. The tissue samples are then digested at 65°C using 40 μl of a solution of activated crystalline papain for several hours. The digest is then quantitatively transferred on the top of a column packed with cellulose powder and saturated with a solution of cetylpyridinium chloride. The precipitated complex is eluted first with 1% cetylpyridinium chloride and then with stepwise increasing concentrations of NaCl or $MgCl_2$, the optimal salt concentrations being selected on the basis of the critical solubility of the expected mucopolysaccharide (41) and tested in pilot experiments. The amount of mucopolysaccharide in each fraction is determined by a slightly modified Elson and Morgan procedure, allowing estimation of about 1 μg of hexosamine as the lower limit. The various possible errors of the procedure, the recovery and the optimal concentrations of the eluents have been determined in experiments using homogenized cartilage powder of horse nasal septa. Such preliminary tests indicated that the main fractions of mucopolysaccharide in this cartilage have their critical solubility in 0.8 and 1.0 *N* $MgCl_2$ respectively, whereas the 1% cetylpyridinium chloride fraction contains considerable amounts of hexosamine.

The method outlined above has been applied to serial sections from a cylinder cut in the cryostat from horse nasal septum, alternate sections

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from a cylinder cut in the cryo-
nasal septum, alternate sections

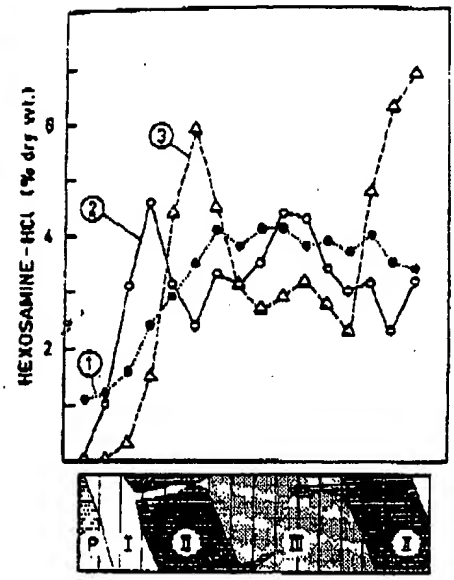

FIG. 5. Quantitative fractionation of mucopolysaccharides on serial frozen sections of the nasal septum cartilage (horse, > 8 years old). The main fractions shown were eluted with 1% cetylpyridinium chloride (1), 0.8 *N* $MgCl_2$ (2) and 1.2 *N* $MgCl_2$ (3). Histological reconstruction of the layers as in Fig. 4. (From Antonopoulos et al., (3).)

being taken for microchemical analysis and histochemical staining. The distribution of the main mucopolysaccharide fractions is plotted against the reconstructed cross section of the nasal septum as shown in Fig. 5. The various fractions follow a different pattern and illustrate marked differences in the composition of the cartilage in various layers. In the subperichondrial part (first layer) the main mucopolysaccharide is contained in the 0.8 *N* $MgCl_2$ soluble fraction, constituting there 60–70% of the total. A few 100 μ inwards, in the second layer, the 1.0 *N* $MgCl_2$ soluble fraction reaches a peak, whereas the former gradually decreases. Both the 0.8 and 1.0 *N* $MgCl_2$ soluble fractions show such a gradual decrease towards the centre, whereas the 1% cetylpyridinium chloride soluble fraction steadily rises. In several experiments with different samples of nasal septa essentially the same pattern was observed, with the 0.8 *N* $MgCl_2$ soluble fraction occupying always a peripheral position compared with the 1.0 *N* $MgCl_2$ soluble fraction, which reached its maximum in the second layer. This was found both in nasal septa of young and old animals. The high 1% cetylpyridinium chloride fraction is characteristic for the third layer, which is only present in certain areas of the nasal septum of old horses (>8 years); in young animals this fraction is uniform in all layers and does not exceed the low values found in the perichondrium.

In several cases such determinations have been made on tissues (nasal septum cartilage, intervertebral disc) separated into the various anatomical layers by microdissection. Such experiments have shown that the mucopolysaccharide composition can differ significantly in these tissues over distances of 100 μ and that such differences can be resolved separating the main mucopolysaccharides into fractions of not more than a few micrograms. The method was also applied to pooled samples of still smaller microscopic dimensions, obtained by microdissection of the chondrones (isogenic chondrocyte groups with the surrounding basophilic zone) and the remaining interterritorial part of the ground substance from the third layer of the nasal septum cartilage. The results are summarized in Table 1 and show that the composition of the mucopolysaccharide in these two areas is essentially identical, and so is the concentration of the total mucopolysaccharide per unit dry weight. This in contrast with the marked differences in histochemical properties of these two structures as apparent from staining with cationic dyes at low pH, whereas at pH 7 no substantial differences in staining intensity could be detected (15, 16).

These results suggest interesting possibilities for the application of such microchemical procedures to the study of various connective tissues. However, several problems obviously still have to be solved, among which first of all that of the further characterization and identification of the

TABLE I

Quantitative Fractionation of the Mucopolysaccharides in the "Chondrone" and "Interterritorial Area" of the Cartilage Ground Substance, Isolated by Microdissection from the Third Layer of the Nasal Septum (Horse, > 8 Years Old)

Eluting Solvent	Hexosamine-HCl (% dry weight)	
	Chondrones	Interterritorial Area
1% CPC	2.9	3.2
0.3 *N* NaCl	0.8	0.7
0.8 *N* $MgCl_2$	2.9	3.0
1.0 *N* $MgCl_2$	1.6	1.2
1.2 *N* $MgCl_2$	0.6	
6 *N* HCl	0.8	0.8
Total	9.4	8.9

mucopolysaccharide fractions. A detailed analysis of a mucopolysaccharide in amounts of a few μg seems not to be feasible with present day methods. Therefore direct conclusions regarding identity of the fractions cannot be easily reached, unless one would rely on the characteristic critical salt solubility itself. On such grounds one could assume that the 0.8 and 1.0 *N* $MgCl_2$ soluble fractions in cartilage would consist of chondroitin sulfates. The 1% cetylpyridinium chloride soluble fraction undoubtedly contains undigested residual components of the tissue such as polypeptides or glycoproteins as well as breakdown products, not precipitated by cetylpyridinium chloride, and probably also keratan sulfate. Keratan sulfate was shown to behave somewhat aberrantly, since its cetylpyridinium chloride-complex—or at least a fraction of it—appears to be soluble in excess of cetylpyridinium chloride (18, 1). The already described presence of a relatively large proportion of glucosamine in the third layer of the horse nasal septum (46, 47) would indicate the possible presence of keratan sulfate, suggesting that this is responsible for the rise of the 1% cetylpyridinium chloride fraction in the central part of this cartilage.

Obviously inferences like above need further proof, which can only be derived from detailed analyses of fractions obtained by the same procedure and under comparable conditions on the macro scale. Such studies are in progress on the nasal septum cartilage and indicate that in fact the 0.8 and 1.0 *N* $MgCl_2$ soluble fractions are likely to be chondroitin sulfate(s): both contain only galactosamine, with hexuronic acid and sulfur in approximately equimolar proportion. In the 1% cetylpyridinium chloride soluble fraction about 90% of the total hexosamine is glucosamine, with an anthrone value (expressed as galactose) and sulfur near to equimolar, compatible with the assumption that the substance is keratan sulfate. While further studies on the identification of these fractions are needed, it would already appear that no simple chemical differences are obvious in the composition of the mucopolysaccharides in the 0.8 and 1.0 *N* $MgCl_2$ soluble fractions. Notable in this connection are results of cartilage analysis in which a further subdivision of these two fractions was obtained, using 0.7, 0.8, 0.9 and 1.0 *N* $MgCl_2$ as eluting solvents. It was interesting to observe that the fraction with the lowest critical solubility (0.7 *N*) occupies the most periferal position and is located immediately underneath the perichondrium, whereas the maximal concentration of the subsequent fraction is located more and more towards the centrum of the nasal septum cartilage. Whatever the explanation of the differences in critical solubility of the cetylpyridinium chloride-complex of these subfractions might turn out to be, they probably represent differences implying some biological significance for the periferal part of the cartilage of the nasal septum.

These preliminary results are intended only to illustrate the possibilities of the microchemical approach along these lines, without concealing the difficulties and limitations. At present the lower limit of the method outlined is in the range of a few μg mucopolysaccharide per fraction, so that the resolution in morphological terms, even with tissues rich in mucopolysaccharide like cartilage, is not more than 50 to 100 μ. Further refinement is required to allow analysis at the cellular level or in tissues with very low mucopolysaccharide content. Our experience with this method is so far limited to hyaline cartilage and the intervertebral disc, and in both cases the digestion procedure applied resulted in complete solubilization of the tissue with no apparent alterations of the mucopolysaccharide. Further studies will have to show whether this procedure will be applicable to other tissues. The resolving power in chemical terms for fractions with very close critical solubility of their cetylpyridinium chloride complexes will also have to be tested in further experiments and ways of identifying the fractions obtained on the microgram scale explored. In spite of many questions to be answered by future work, the results already obtained with this method illustrate its potential value for quantitative histochemistry.

SUMMARY AND CONCLUSION

Quantitative histochemistry of mucopolysaccharides, although in the early beginnings at present, seems to be practicable along two lines discussed in this review. The interaction of cationic dyes with mucopolysaccharides appears to be of quantitative and stoichiometric nature and validates the quantitative evaluation of the staining with cationic dyes if certain conditions are fulfilled. Fixation in the customary sense interferes with the demonstration of mucopolysaccharides with cationic dyes in several ways and can be avoided through direct *in situ* precipitation of the mucopolysaccharides with cationic dyes. Under carefully controlled conditions quantitation of the free anionic groups of mucopolysaccharides might be applicable to microspectrophotometry on the basis of the stoichiometry of this reaction. A quantitative measure of the total free anionic groups in a tissue can be achieved by estimation of the dye-uptake

in vitro, and under certain conditions can provide a reasonably accurate estimate of the total mucopolysaccharide content. In neither of these two approaches the use of cationic dyes offers a simple means of quantitatively distinguishing the various types of mucopolysaccharide. This goal seems to be more easily attainable at present by application of chemical methods on the microgram scale, as described here on the basis of fractional elution of mucopolysaccharide-cetylpyridinium complexes and enabling determination of a few micrograms of mucopolysaccharide in a given fraction from digested samples of tissues like cartilage and intervertebral disc. Such microchemical methods are expected to offer many possibilities in analytical and metabolic studies of mucopolysaccharides on the tissue level; they also may be used to check the validity of histochemical methods of the staining type.

Acknowledgments. The approaches summarized in this review ripened through periods of fruitful collaboration with Dr. E. A. Balazs (Boston) and Dr. S. Gardell (Lund), whose interest for the histochemical level of analysis was most stimulating. The cooperation during various phases of these studies with J. Doyle, B.Sc., Dr. H. Galjaard, F. Klein, chem.drs., Miss M. T. C. Knaap, Miss P. C. van der Linde and Miss E. R. de Tyssonsk is gratefully acknowledged. Financial support for various parts of the original work reported was obtained in part by grants of the U. S. Public Health Service (A-2108), the Helen Hay Whitney Foundation and the Rheumatism Research Foundation "Dr. Jan van Breemen" and by fellowships or travel grants from the Retina Foundation and the Netherlands Organization for Pure Research.

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